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Award Number: DAMD17-98-1-8477

TITLE: Molecular Basis of Prostate-Specific Androgen-Independent Expression of a Homeobox Gene

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REPORT DATE: April 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2001	Annual (13 Sep 99 - 12 Mar 01)

4. TITLE AND SUBTITLE
Molecular Basis of Prostate-Specific Androgen-Independent Expression of a Homeobox Gene

5. FUNDING NUMBERS
DAMD17-98-1-8477

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The goal of the work supported by this grant is to characterize the molecular basis of prostate specificity and androgen independence of the *hoxb-13* gene with a view towards developing new treatments for advanced prostate cancer. To date, we have succeeded in generating a physical map of both the mouse and human hoxb-13 loci, and have sublconed regions both upstream and downstream of the coding region of both genes. Reporter gene constructs carrying up to 12.5 kb of the human *hoxb-13* locus have been completed and transgenic mice carrying these constructs have been generated. Analyses of reporter gene expression in these mice have revealed that this region contains elements capable of directing a pattern of gene expression that recapitulates features of *hoxb-13* expression but does not direct expression in the prostate gland. Transgenes carrying regions farther upstream as well as downstream of *hoxb-13* loci are being constructed to widen the search for cis-acting elements that function in the prostate.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Prostate	13
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

Hox genes encode the transcriptional regulatory proteins that are largely responsible for establishing the body plan of all metazoan organisms. A subset of Hox genes continues to be expressed during the period of organogenesis and into adulthood. *Hoxb-13* is a member of the Hox gene family that is expressed in the spinal cord, hindgut, and urogenital sinus during embryogenesis. We have characterized its expression in adult mouse tissues and have found that it is expressed in only two sites: the prostate gland and the distal colon. Surprisingly, accumulation of *Hoxb-13* mRNA is not diminished in prostate glands following castration indicating that its expression is androgen independent. In support of this suggestion, we have also demonstrated that the human *Hoxb-13* gene is expressed in androgen-independent PC-3 cells, as well as androgen-starved LNCaP cells. Stimulation of LNCaP cells with androgen does not alter the expression of *Hoxb-13*. In our Phase I application, we proposed to characterize the molecular basis of prostate specificity and androgen independence of the *Hoxb-13* gene with a view towards developing new treatments for advanced prostate cancer. We have characterized a P1 artificial chromosome that contains the human *Hoxb-13* locus and have subcloned a total of 30 kb. Several fragments from this region were refractory to cloning in conventional plasmid vectors, and we were unexpectedly required to develop a new vector in which these fragments could be propagated. Reporter gene analyses have been completed in a series of transgenic mice, and we have succeeded in identifying a region of the human *Hoxb-13* gene that contains cis-acting control elements that direct a pattern of expression in embryos that parallels the endogenous mouse *hoxb-13* gene. However, these elements are not sufficient to direct expression in adult prostate epithelial cells. These data demonstrate that the 15 kilobase region that has been analyzed to date contains a functional *Hoxb-13* promoter and the enhancer elements that can dictate an appropriate pattern of expression in embryos. This work has brought us a step closer to the identification of androgen-independent regulatory elements that support gene expression in the prostate gland. These elements would provide a unique reagent that could be incorporated into gene therapy strategies to treat advanced prostate cancer in patients that have undergone androgen deprivation therapy. Currently available prostate-specific regulatory elements are all androgen dependent and would be unlikely to function well in these patients.

BODY:

Task 1. To identify a region of the human *Hoxb-13* locus that is capable of directing expression of a reporter gene to prostatic epithelial cells in an androgen independent manner (months 1-30).

Identification of cis-acting regulatory elements of the human Hoxb-13 gene that direct expression in mouse embryos. As outlined in our phase I proposal, our initial efforts to locate prostate transcriptional control elements of *Hoxb-13* focused on sequences upstream of the coding region. The initial construct contained 8 kb of DNA extending from the translation start site to an upstream EcoRV restriction site. This construct was injected into FVB embryos, and 6 transgenic lines carrying the transgene were established. Analyses of lacZ reporter gene activity in transgenic embryos revealed strong reporter gene activity in a pattern that recapitulated the major aspects of the expected pattern for *hoxb-13* in 9.5-12.5 days post coitum (dpc) embryos (Figure 1 and data not shown). Strong ß-galactosidase (ß-gal) activity was observed in 9.5 dpc embryos in posterior somites, unsegmented mesoderm, and the developing spinal cord, in concordance with the pattern predicted by in situ hybridization studies. This pattern continued in 10.5 dpc embryos, however, ectopic expression was also observed in the myotome portion of somites anterior to the normal

domain of *hoxb-13* expression. This observation indicates that the construct may lack a negative regulatory element that would preclude expression in the myotome. Although the intensity of staining varied among the three transgenic lines, the pattern was remarkably consistent. Deletion of the distal 4 kb from this transgene resulted in a construct that was not expressed in the expected *hoxb-13* pattern, and was instead active in a few cells in a pattern that varied from embryos to embryo (data not shown).

At 12.5 dpc, expression of the endogenous *hoxb-13* gene is observed in the developing urogenital sinus. Surprisingly, analyses of ß-gal activity in transgenic embryos carrying the 8 kb upstream construct failed to show reporter gene activity in the urogenital sinus region in both whole mount preparations and in histological sections. Analyses of ß-gal activity in neonatal and adult prostate glands also failed to show measurable reporter gene activity. Consistent with these observations, Northern blot analyses of prostate RNA extracted from adult transgenic mice demonstrated that the reporter gene mRNA was not detectable (data not shown).

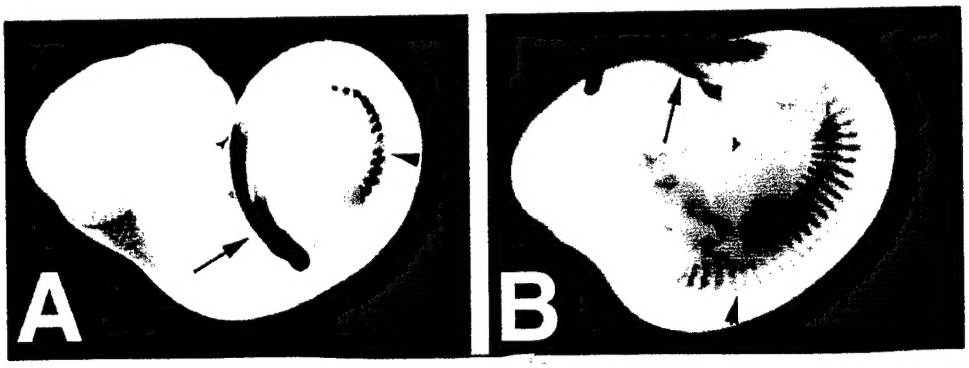

Figure 1. ß-gal staining of embryos carrying the 8 kb *Hoxb-13*/lacZ transgene. A, 10.5, B, 11.5 dpc embryo. Arrow points to posterior expression in the spinal cord and mesoderm. Arrowhead denotes ectopic expression in the myotome and its derivatives.

Expanding the search for hoxb-13 cis acting control elements. The lack of prostate expression in transgenic mice carrying the 8 kb/lacZ construct prompted us to expand the search for cis-acting control elements by adding sequences both farther upstream as well as downstream of the *Hoxb-13* translational start site. The strategy we adopted was to insert the lacZ gene near the translational start site in a fragment that contained an additional 2.5 kb of upstream sequences and 2 kb of DNA downstream of the start site. At this juncture, our efforts to further characterize *hoxb-13* cis acting elements were slowed by cloning difficulties.

The 10.5 kilobase EcoRI-BspHI fragment that was our target for the next lacZ transgene proved to be refractory to cloning in a series of conventional plasmid vectors and bacterial strains of various combinations. Vectors employed included pBluescript (Stratagene), pGEM-Teasy (Promega), and pZero-2 (Invitrogen). Bacterial strains included supercompetent DH10B, XL1-Blue, and XL-10Gold. These experiments failed in the hands of a research associate with more than 12 years of cloning experience, and other constructs with similar insert sizes were routinely generated during the same 12 month period.

Hypothesizing that the bacterial host could perhaps tolerate the *Hoxb-13* sequences in a lower dose, we attempted to clone the same fragment in a vector termed pACYC184. This plasmid, which has a p15A plasmid origin of replication, is a "medium" copy number plasmid that does not contain a polylinker region. Recombinants containing the *Hoxb-13* sequences that were "unclonable" in conventional vectors were obtained on the first attempt using pACYC184 as a vector. While these results allowed us to overcome the refractory-to-cloning impasse, weencountered new challenges in designing transgenes based on this vector. Due to the well-documented negative effects of plasmid sequences on gene expression in transgenic mice, it is imperative to remove vector DNA from the fragment to be injected into embryos. The lack of a polylinker in pACYC184 made this critical requirement extremely difficult if not impossible.

Generation of a new transgene vector. To allow us to take advantage of the fact that pACYC184 was capable of supporting the replication of *Hoxb-13* sequences, we endeavored to modify the vector to incorporate a polylinker geared specifically to house mouse transgenes. To this end, we designed a polylinker that contained 13 centrally located unique 6-base restriction sites, flanked by a series of 8-base restriction sites, two of which were duplicated to allow for one-step removal of vector sequences (Figure 2). The extreme 5' and 3' ends of the polylinker contain a 34-base recognition site for an intron-encoded endonuclease, which can also be used to release vector sequences if the 8-base sites occur within the transgene insert (Figure 2). This polylinker was generated using a PCR SOEing strategy, and was inserted into a derivative of pACYC184 that had been reduced in size by elimination of 2 kb of unnecessary sequences. The resulting polylinker was sequenced to confirm its authenticity and the lacZ gene was inserted into its center. This effort, though costly in terms of time, was absolutely required for us to move forward in the search for cis-acting sequences from the human *Hoxb-13* gene.

Activity of a 12.5-kb Hoxb-13/lacZ transgene. The pACYC184-based construct containing lacZ inserted at the translational start site within a 12.5 kb region of the *Hoxb-13* locus was injected into FVB embryos to derive transgenic mice. The impending date for submission of this phase II proposal precluded us from analyzing transgenic F1 mice carrying this construct, so we opted instead to euthanize founder transgenic mice to characterize gene expression during embryogenesis and in the prostate gland of postnatal mice. Analysis of one litter of 8 embryos arising from fertilized eggs injected with this construct showed one 10.5 dpc embryo with ß-gal activity in the anticipated *Hoxb-13* pattern (data not shown). Ectopic expression of the lacZ gene in myotomes was not observed in this embryo. This observation confirmed that the transgene was functional and capable of expressing the lacZ gene and that a negative regulatory element that suppresses ectopic expression in somites was present.

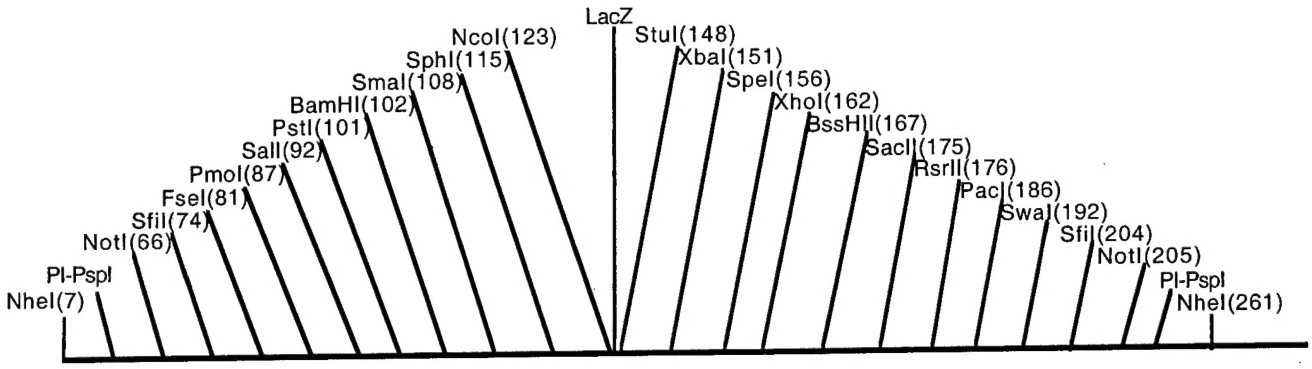

Figure 2. Polylinker region of the modified pACYC-184 vector. The total length of the polylinker is 270 bp.

Of 64 founder generation animals born, 4 female and 4 male transgenics were identified by Southern blot analysis. Following prostate dissection, half of each gland was processed for analysis of ß-gal activity and RNA was extracted from the other half. Posterior colon (including rectum) and seminal vesicles were also harvested and processed similarly. Identical sets of tissue from non-transgenic littermates served as negative controls. Whole mount staining of tissues from transgenic and non-transgenic mice failed to reveal ß-gal activity in the transgenic prostate glands that was measurable above the background level of endogenous ß-gal activity (data not shown). In addition, Northern blot analyses of RNA extracted from transgenic tissues failed to detect lacZ mRNA.

KEY RESEARCH ACCOMPLISHMENTS

- analysis of reporter gene expression in mice carrying 8 kb of hoxb-13 sequences
- generation of a modified pACYC184 vector
- cloning of a 12.5 kb hoxb-13 region that was refractory to cloning in conventional vectors
- construction of a 12.5 kb hoxb-13/lacZ transgene
-generation of transgenic mice carrying 12.5 bk hoxb-13/lacZ transgene
-analysis of reporter gene expression in mice carrying 12.5 kb hoxb-13/lacZ transgene

REPORTABLE OUTCOMES:

Manuscripts

Sreenath T, Orosz A, Fujita K, Bieberich CJ. (1999) Androgen-independent expression of *hoxb-13* in the mouse prostate. Prostate 41:203-7.

CONCLUSIONS:

Although we have succeeded in identifying a promoter activity from the human *Hoxb-13* locus that directs expression in mouse embryos, we have yet to identify the cis-regulatory element(s) that are capable of directing expression in the prostate gland. Our efforts were temporarily bogged down by the difficulties we encountered in cloning certain fragments of the *Hoxb-13* locus. We suspect that we are not alone in being unable to clone these regions in conventional vectors: we have recently sequenced the ends of our pACYC184-based *Hoxb-13* clones and have BLASTed them against the deposited human genome sequence. Despite the fact that a "rough draft" of the human genome is nearly complete, the Hoxb-13 locus sequence from our pACYC184 clones is not present. We remain committed to identifying the elements required to direct androgen-independent gene expression in the prostate gland. These regulatory elements would be immediately useful in gene therapy approaches to treat advanced prostate cancer, and may shed light on the molecular basis of androgen-independent gene expression in prostate epithelial cells.

REFERENCES:

N/A

APPENDICES:

Reprint- Prostate 41:203-207 (1999).

The Prostate 41:203–207 (1999)

Rapid Communication

Androgen-Independent Expression of *hoxb-13* in the Mouse Prostate

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BACKGROUND. Hox genes encode transcriptional regulatory proteins that are largely responsible for establishing the body plan of all metazoan organisms. A subset of Hox genes is expressed during the period of organogenesis and into adulthood. *hoxb-13* is a recently-described member of the Hox gene family that is expressed in the spinal cord, hindgut, and urogenital sinus during embryogenesis.
METHODS. Northern blot and in situ hybridization analyses of *hoxb-13* expression in adult mouse tissues were performed.
RESULTS. *hoxb-13* mRNA is restricted to the prostate gland and distal colon in adult animals. In situ hybridization of mouse prostate tissue demonstrated that *hoxb-13* is expressed in the epithelial cells of the ventral, dorsal, lateral, and anterior prostate lobes. Accumulation of *hoxb-13* mRNA is not diminished following castration.
CONCLUSIONS. These data demonstrate that *hoxb-13* expression is androgen-independent in mouse prostate glands. The identification of *hoxb-13* as an androgen-independent gene expressed in adult mouse prostate epithelial cells provides a new potential target for developing therapeutics to treat advanced prostate cancer. *Prostate 41:203–207, 1999.*

KEY WORDS: **homeobox gene; gene expression; castration; cancer**

INTRODUCTION

The mammalian Hox genes are homologs of *Drosophila* homeotic genes that encode homeodomain transcription factors [1]. Gain and loss of function analyses have demonstrated that Hox genes in mammals play a critical role in establishing the basic body plan, for example, by patterning the axial and the appendicular skeleton and parts of the central and peripheral nervous systems during embryogenesis [1–3]. An intriguing feature of some Hox genes is their continued expression during organogenesis and in differentiated organs in adult animals [1]. Although their roles in pattern formation during early embryonic development have been studied extensively, their roles in later developmental events and tissue maintenance have received comparatively little attention. The importance of understanding their functions in differentiated cells is underscored by the recognition that deregulated expression of Hox and other classes of homeobox-containing genes have been implicated in oncogenic transformation of cultured cells and in tumors [4–9].

Several members of the Hox family of homeobox genes have been found to be expressed in human prostate cell lines [6] and in mouse embryos in the region of the urogenital sinus that gives rise to pros-

Grant sponsor: U.S. Army Medical Research and Materiel Command Prostate Cancer Research Program; Grant number: DAMD17-98-1-8477.

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Received 10 June 1999; Accepted 18 August 1999

tate glands [10,11]. *hoxd-13* has been shown to be expressed in the urogenital sinus during late gestation and in early postnatal mouse prostate glands, but is repressed at maturity [10]. A loss-of-function mutation of *hoxd-13* in mice results in a decrease in size and ductal branching of the dorsal and ventral prostate lobes [12]. Both *hoxc-11* and *hoxb-13* have been reported to be expressed in the prostatic region of the urogenital sinus at 12.5 days gestation, but their distribution at later stages and in adult tissues have not been described [11,13].

We have characterized the distribution of *hoxb-13* mRNA in adult mouse tissues and have found that it is expressed at a high level in the prostate gland and distal colon.

MATERIALS AND METHODS

RNA extractions and Northern blot analyses were performed essentially as described [14] using either a 4.5 kb probe containing the entire *hoxb-13* coding region (T.S. and C.J.B., unpublished) or a 550 bp *Apa*I-*Eco*RI restriction fragment containing only exon 1. Identical results were obtained with both probes. Dissection of prostate glands into component lobes was performed as described [15]. Orchiectomy was carried out on 6-week-old CD-1 mice as described for rats [16], and RNA was extracted from whole prostate glands pooled from two mice at each time point. In situ hybridization was performed as described [17] using 0.45 kb *Bgl*II-*Xba*I fragment derived from the 3′ end of *hoxb-13* [13].

RESULTS AND DISCUSSION

To determine whether *hoxb-13* is expressed in adult tissues, Northern blot analyses were performed. A 4.5 kb probe derived from the *hoxb-13* genomic locus and containing the entire transcription unit was hybridized to total RNA extracted from 18 different tissues of adult FVB mice (Fig. 1 and data not shown). A strongly hybridizing 3.2 kb component was observed only in RNA from the prostate and the distal region of the colon (Fig. 1A,B). Within the prostate gland, *hoxb-13* mRNA was detected in all lobes (Fig. 1A). When normalized to the level of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the ventral and lateral lobes showed a similar steady-state level of expression, while in the coagulating gland (anterior prostate) and dorsal prostate the level was consistently 2-3 fold lower. A distal-to-proximal gradient of *hoxb-13* mRNA was observed in the colon (Fig. 1B). The rectum and 1 cm of adjacent colon showed the highest level of expression, while in the most proximal 1 cm of colon, just distal to the cecum,

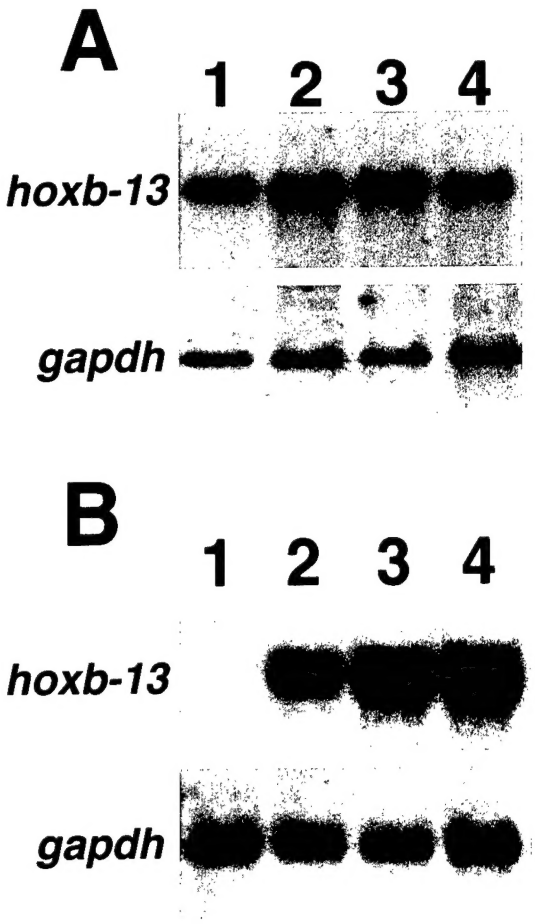

Fig. 1. Northern blot analysis of *hoxb-13*m RNA expression in adult mouse tissues. Panel **A**: lane 1, coagulating gland; lane 2, ventral prostate; lane 3, lateral prostate; lane 4, dorsal prostate. Panel **B**: lane 1, proximal colon, defined as 1 cm of intestinal tissue just distal to the cecum; lane 2, middle colon, defined as the central 1 cm of tissue between the distal margin of the cecum and the anus; lane 3, distal colon, defined as 1cm of colon adjacent to the rectum; lane 4, rectum. Average length of colon from the distal margin of the cecum to the anus was 8 cm.

hoxb-13 mRNA was not detected (Fig. 1B). *hoxb-13* mRNA was also detected in the central region of the colon at a level two-fold lower than in the rectum (Fig. 1B). Expression of *hoxb-13* was not detected by Northern blot analysis in other urogenital tissues including kidney, testis, urethra, bladder, seminal vesicle, ampullary gland, vas deferens, ovary, or uterus (data not shown). *hoxb-13* expression was also not detected in RNA from preputial gland, liver, spleen, lung, heart, thymus and brain (data not shown).

To characterize the cellular distribution of *hoxb-13* mRNA within the mouse prostate gland, in situ hybridization analyses were performed on histological sections of adult tissue. As predicted by the Northern blot analyses, hybridization to the *hoxb-13* probe was observed in all prostate lobes (Fig. 2 and data not

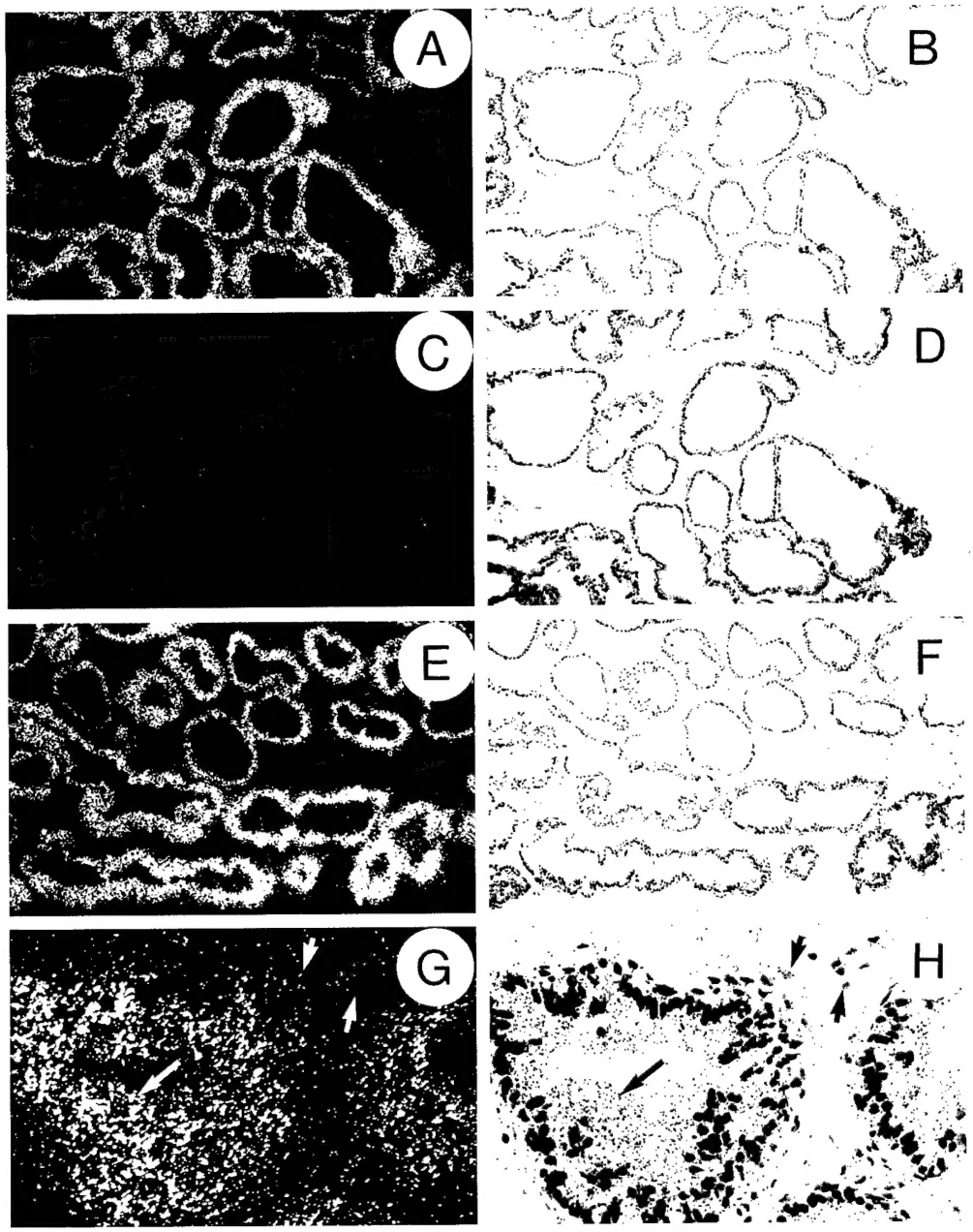

Fig. 2. In situ hybridization analysis of *hoxb-13* expression in adult prostate. Paraffin sections of ventral (A-D) and lateral (E-H) lobes. **A**, darkfield, **B**, brightfield low magnification view shows signal over ducts in the ventral lobe using the antisense probe. **C**, darkfield, **D**, brightfield view shows lack of signal with a sense probe on a serial section, demonstrating the specificity of the assay. **E**, darkfield, **F**, brightfield low magnification view of lateral lobe ducts. **G**, darkfield, **H**, brightfield high magnification view of a lateral duct shows hybridization signal over epithelial cells. Short arrows highlight stromal cells, long arrow points to epithelial cells.

shown). Hybridization signal was seen along the length of the ducts but was excluded from the proximal region of each main duct near the junction with the urethra. Within the ducts, strong hybridization signal was observed over luminal epithelial cells, while stromal cells did not display signal that was distinguishable from background.

The effect of castration-induced androgen deprivation on the steady-state level of *hoxb-13* mRNA was examined by Northern blot analysis. Surprisingly, the level of *hoxb-13* mRNA was not diminished within 8 days after orchiectomy when normalized to the level of GAPDH (Fig. 3 and data not shown). In contrast, expression of *mp12*, an androgen-dependent gene which encodes a protease inhibitor, decreased 70-fold 24 hours after castration and was not detectable after

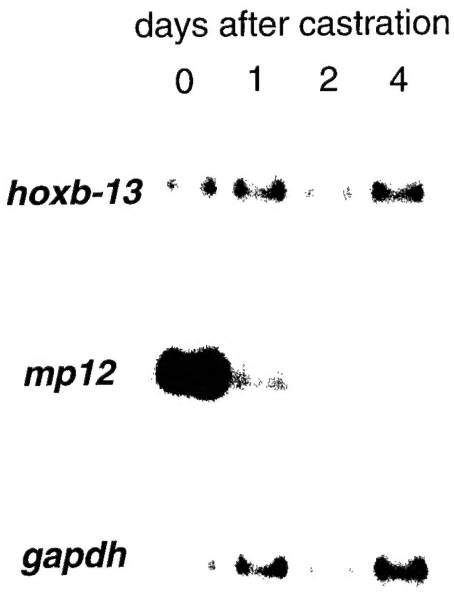

Fig. 3. Northern blot analysis of *hoxb-13* expression in orchiectomized mice. The same Northern blot membrane was sequentially hybridized with the indicated probes.

48 hours (Fig. 3) [18]. Similarly, the level of mRNA encoded by *nkx-3.1*, a homeobox gene that has been demonstrated to be androgen-dependent, was decreased nearly 10-fold within 24 hours, and 30-fold after 4 days (data not shown). These data indicate that maintenance of a high steady-state level of *hoxb-13* mRNA in the prostate gland does not require testicular androgens. It has been demonstrated in rats that a significant proportion (~20%) of ventral prostate epithelial cells survive at least 1 week after castration [19]. Our observations suggest that *hoxb-13* expression persists, and may even be up-regulated in the mouse prostatic epithelial cells that survive after castration.

Despite the fact that it is the most diseased organ in the human body, the genetic basis of prostate development, differentiation, and maintenance is not well understood. Considerable effort is currently focused on defining the role of androgens and peptide growth factors in prostate development and disease [20]. However, the nature of the genetic program(s) active in epithelial cells that underlie differentiation and maintenance remain largely undefined. Recently, several homeobox genes have been strongly implicated in both normal and malignant growth of prostate epithelial cells. The mouse *nkx-3.1* homeobox gene has been found to be expressed in developing and mature prostate glands where it is restricted to ductal epithelial cells and is androgen-dependent [14,21,22]. *nkx-3.1* is the earliest known marker of prostatic epithelial cells, and a loss-of-function mutation results in reduced ductal branching in all prostate lobes as well as defects in secretory protein production [23]. *nkx-3.1* null mice also develop epithelial hyperplasia and dysplasia in the anterior and dorsolateral lobes that increases in severity with age, suggesting that this homeobox gene may be a candidate tumor suppressor gene [23]. In contrast, the homeobox genes GBX1 and GBX2 have been found to be overexpressed in prostate cancer cell lines, and reduction of GBX 2 expression results in decreased clonogenic ability in vitro and tumorigenicity in vivo [6]. Together these data suggest that both gain and loss of function of certain homeobox genes may play a role in prostate cancer progression. Interestingly, human *hoxb-13* expressed sequence tags have been observed in both prostate and colon carcinomas [24].

Although most prostate tumors initially respond to androgen deprivation therapy, nearly all return as androgen-independent disease. Currently, there is a dearth of effective treatment options available to patients with advanced prostate cancer. The need to identify new potential targets for therapeutic intervention and to develop novel therapies to treat androgen-independent prostate cancer is axiomatic. Our observation of androgen-independent expression of *hoxb-13* in the mouse prostate provides a new model to study the molecular basis of androgen-independent gene expression in prostate epithelial cells.

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